The Hemocyanins of *Tegula funebralis* and *Tegula brunnea*

(Mollusca : Gastropoda)

BY

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(3 Text figures)

THE PURPOSE OF THIS STUDY was to develop a method of purification of hemocyanin and to compare some properties of this respiratory pigment in *Tegula funebralis* (A. ADAMS, 1854) and *Tegula brunnea* (PHILIPPI, 1848).

METHODS AND MATERIALS

Animals were collected at Mussel Point and at Point Pinos, Pacific Grove, California. After one hour of anesthesia, in 0.01% (w/v) solution of "Sevin 50" (1-napthyl-N-methyl carbamate) in filtered sea water, the foot of the animal, which becomes somewhat distended, was lanced and the blood collected in a small crucible. Care was taken in collecting the blood to prevent contamination with fluid from the mantle cavity. Contamination was minimized by shaking the animal in order to remove most of this fluid and by holding the foot of the animal between thumb and forefinger while lancing to prevent the animal from withdrawing into its shell and forcing the fluid out. The blood was centrifuged for 30 minutes to remove cells and other debris, and either analyzed immediately or frozen for study at a later time. Freezing produced no noticeable effect on the blood.

The oxygen dissociation data were obtained on 5 ml samples of blood placed in a Klett tube and covered with a vaccine cap. Different mixtures of air and nitrogen were regulated with two Gelman flowmeters (range 1 to 10 CFH) and allowed to pass over the sample of blood. The gas input and exhaust were equipped with 20-gauge hypodermic needles. In order to prevent excessive evaporation of the sample, the gas mixture was only passed over it for 10 minutes. The sample was then shaken to equilibrate it with the gas mixture in the sealed tube, and the density of color measured in a Klett-Summerson Photoelectric Colorimeter using a no. 54 green filter (520 to 580 mμ) against a blank of distilled water. The process was repeated until a constant reading was obtained for any gas mixture. Values were obtained for varying ratios of air to nitrogen starting with pure nitrogen in which hemocyanin became completely reduced and ending with pure air in which the pigment became fully oxygenated. Values for reduced and oxygenated hemocyanin were obtained for various dilutions of the blood in filtered sea water adjusted to the same pH as the blood in order to establish a standard curve for concentration of oxidized *Tegula* hemocyanin.

Purification of the hemocyanin was accomplished by filtration of samples of blood through a 6 cc Sephadex G-200 column. Initial fractionation of 1 ml samples of blood into 1 ml aliquots was evaluated by observing the location of the blue color characteristic of hemocyanin and measurement of the protein by the method of LOWRY (1951) using a standard curve prepared with Armour Bovine Serum Albumin Fraction V. The void volume of

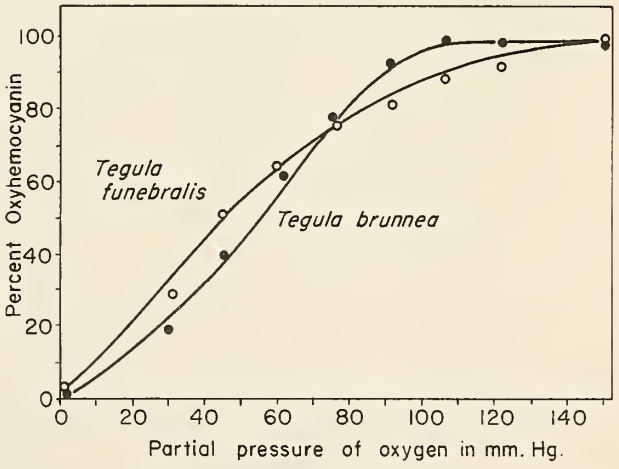

Figure 1: Oxygen dissociation curves for *Tegula funebralis* and *Tegula brunnea*. pH for *T. funebralis* is 7.2 and for *T. brunnea* is 7.8. Temperature for both species is 15° C. Values for *T. funebralis* are represented by open circles and for *T. brunnea* by closed circles.

the column was established using 1 ml of a mixture of diluted india ink and phenol red dye. Samples of hemocyanin thus purified were used for further study.

The nitrogen content was determined by Nesslerization after digestion in H_2SO_4, H_3PO_4 1 : 3 containing 0.55% $CuSO_4$. Dry weight was determined on samples after drying in an oven at 80° C to constant weight.

The absorption spectrum was determined on a Beckman DKB spectrophotometer.

RESULTS

Oxygen equilibrium curves are shown in Figure 1. Both curves were corrected for the absorption and light scattering by the use of a completely reduced sample of blood. The pH of *Tegula funebralis* blood was 7.2 and the temperature for gas equilibration was 15° C. The P_{50} for the sample, i. e., the partial pressure at which 50% saturation of the hemocyanin was reached, was 45 mm Hg. The pH of *T. brunnea* blood was 7.8 and equilibration carried out at the same temperature revealed a P_{50} of 55 mm Hg.

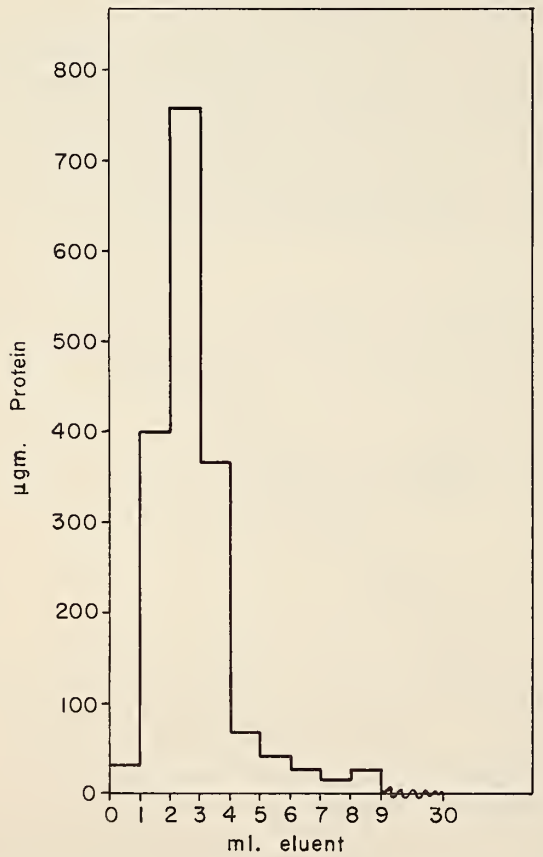

Figure 2: Fractionation of *Tegula brunnea* serum by Gel filtration through Sephadex G-200. Elution with 3% NaCl

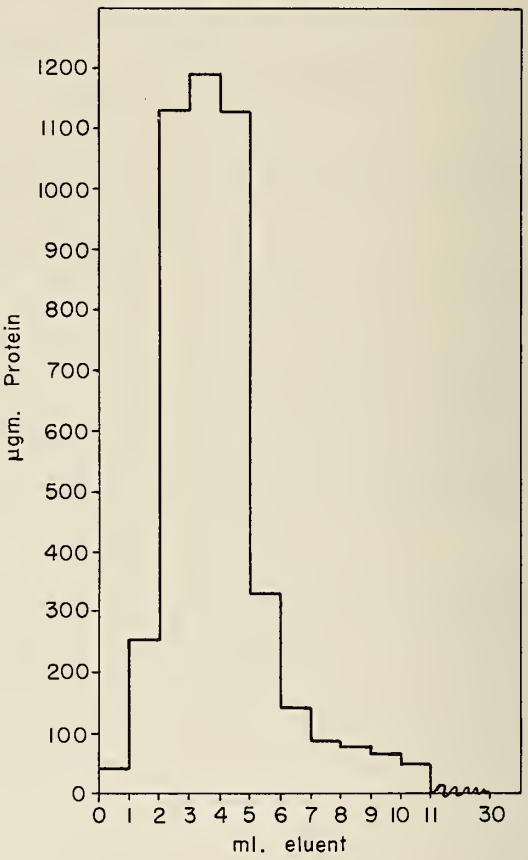

Figure 3: Fractionation of *Tegula funebralis* serum by Gel filtration through Sephadex G-200. Elution with 3% NaCl.

In order to investigate the nature of interaction between prosthetic groups of the pigment, the Hill equation for respiratory pigments was used (MANWELL, 1960, p. 207). For *Tegula funebralis* and *T. brunnea* at the pH used, the line obtained from plotting the Hill equation logarithmically is curved. Hence, interaction between prosthetic groups varies over the range of oxygen tensions.

Figures 2 and 3 show the results of fractionation of the blood of *Tegula funebralis* and *T. brunnea* on a Sephadex G-200 column. The characteristic blue color of oxyhemocyanin could be detected in the third, fourth, and fifth ml of eluent for *T. funebralis* and second, third, and fourth ml of eluent for *T. brunnea*. These were coincident with the elution pattern in terms of protein. The void volume of the column corresponded to the first two ml of eluent. The elution of the pigment, as was expected, indicates a

molecular size in excess of that corresponding to a molecular weight of 200000.

The nitrogen content of the purified hemocyanin corresponded to the expected amount in terms of the Lowry protein estimation. The nitrogen content in terms of per cent dry weight was approximately 15%. Therefore, the purified material was not contaminated with nitrogen-containing non-protein material nor with large molecular weight material lacking nitrogen.

The purified material gave the two peaks characteristic of hemocyanin. The broader minor peak had a maximum at 560 mμ and the sharper major peak at 371 mμ.

DISCUSSION

Since the oxygen dissociation curves are based on the examination of only two samples, an interpretation of the small differences observed is not possible.

The use of Sephadex G-200 for purification of hemocyanin produces very clean samples of the pigment in any desired solution of electrolyte. Fractionation of the blood of *Tegula funebralis* and *T. brunnea* indicates that the molecular size of these hemocyanins exceeds that of a molecule with a molecular weight of 200000. This agrees with the values listed in the literature (Redfield, 1950). It appears that there are no other smaller molecular weight proteins or polypeptides in the blood of these animals since only the one peak appears on elution.

SUMMARY

1. Hemocyanins from *Tegula funebralis* and *T. brunnea* were compared with respect to oxygen equilibration.
2. Only slight differences between the hemocyanins for the two species were detected in the oxygen dissociation curves.
3. P_{50} values for *T. funebralis* and *T. brunnea* were determined to be 45 and 55 mm Hg respectively.
4. For the pH used, prosthetic group interaction appeared to vary depending on the oxygen partial pressure.
5. A method for purification of hemocyanin was developed using Sephadex G-200.
6. It appears that no smaller molecular weight proteins or polypeptides are present in these sera.
7. The absorption spectrum for *T. funebralis* blood gives the major peak at 371 mμ and the minor peak at 560 mμ.

LITERATURE CITED

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